

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

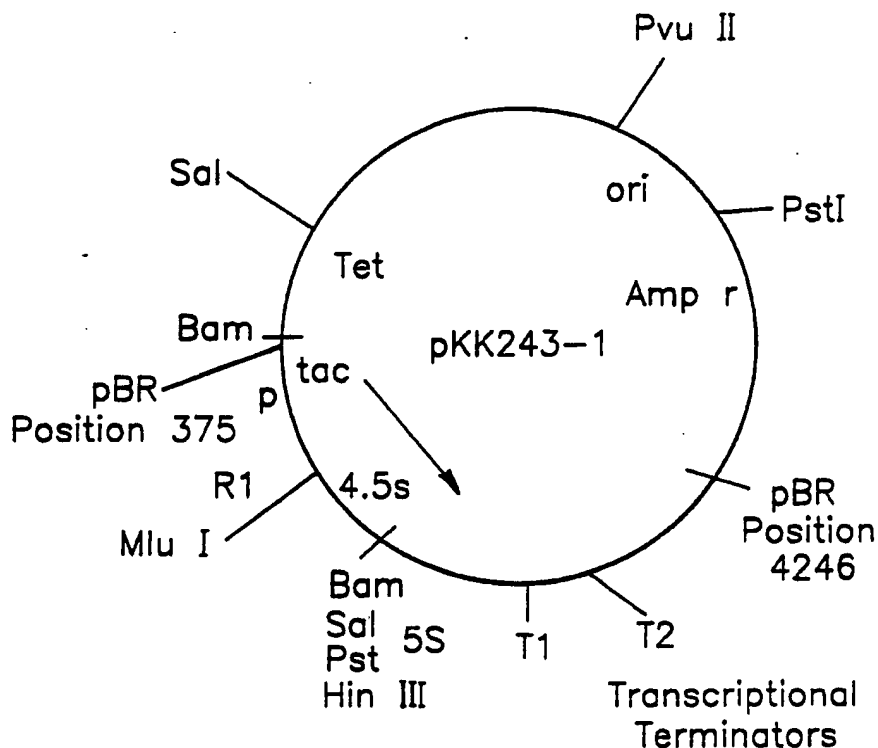
**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C07H 15/12, C12Q 1/68 C12P 21/06, 21/02, 21/04 C12P 19/34, C12N 15/00, 1/20</p>	<p>A1</p>	<p>(11) International Publication Number: WO 92/06988</p> <p>(43) International Publication Date: 30 April 1992 (30.04.92)</p>
<p>(21) International Application Number: PCT/US90/06032</p> <p>(22) International Filing Date: 19 October 1990 (19.10.90)</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 401,613 (CIP) Filed on 31 August 1989 (31.08.89)</p> <p>(71) Applicant (for all designated States except US): CITY OF HOPE [US/US]; 1500 East Duarte Road, Duarte, CA 91010-0269 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : ROSSI, John, J. [US/US]; 346 Cimмерon Trail, Glendora, CA 91740 (US). TAYLOR, Nerida [AU/US]; 25337 Cypress Street, Loma Linda, CA 92354 (US).</p>		<p>(74) Agent: IRONS, Edward, S.; 919 - 18th Street, N.W., Suite 800, Washington, DC 20006 (US).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</p> <p>Published With international search report.</p>

(54) Title: METHOD FOR THE PRODUCTION OF CATALYTIC RNA IN BACTERIA



(57) Abstract

E. coli transformed with genes which express anti-HIV-1 ribozymes. Mammalian cells which express HIV-1 *gag* ribozymes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU ⁺	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE*	Germany	MC	Monaco	US	United States of America
DK	Denmark				

⁺ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

-1-

METHOD FOR THE PRODUCTION
OF CATALYTIC RNA IN BACTERIA

This application is a continuation-in-part of Rossi, Cantin, Zaia and Chang United States application Serial No. 07/369,489 filed 21 June 1989, and of Rossi, Cantin, Zaia and Chang United States application Serial No. 07/401,613 filed 31 August 1989 as a continuation-in-part of Serial No. 07/369,489. Application Serial No. 07/369,489 and application Serial No. 07/401,613 are incorporated herein by reference.

This invention relates to a method for the production of catalytic RNA (ribozymes) in cells, including mammalian and bacterial cells. More particularly, the invention relates to synthetic genes which encode a ribozyme, to mammalian and bacterial cells having such genes transformed therein, and to the ribozyme containing expression products of such bacteria. The invention also includes the in vitro and therapeutic use of such expression products.

BACKGROUND OF THE INVENTION

One form of gene expression impairment by RNA-RNA duplex formation has been termed "antisense" inhibition. Exploitation of antisense gene regulation could lead to potent anti-viral therapy. A serious limitation of the antisense approach, especially as it applies to anti-viral activity, is that it is stoichiometric and may require large molar excesses of anti-sense versus target RNA to be effective.

-2-

Within recent years, discoveries of ribozymes, e.g., RNAs with enzymatic activities have led to the development of antisense molecules which not only form RNA-RNA hybrids, but catalytically cleave the covalent phosphodiester linkages and turn over large numbers of substrate molecules. Ribozymes can now be targeted to virtually any RNA transcript, and efficient cleavage can be readily achieved in vitro. See, Kim, S.H., et al. Proc. Natl Acad. Sci. U.S.A. 84:8788-8792 (1987); Haseloff, J., et al., Nature 234:585-591 (1988); PCT published application WO/89/05852; Cech, T.R. JAMA 260:3030-3034 (1988); PCT published application WO/88/04300; Jeffries, A.G., et al., Nucleic Acids Research 17:1371-1377 (1989).

Applications Serial No. 369,489 and Serial No. 401,613 describe stable, catalytically efficient ribozymes useful, inter alia, to cleave HIV-1 RNA or any other viral or endogenous cellular RNA in vitro and in vivo, mammalian cells transformed with such ribozymes, vectors useful to accomplish such transformation and the use for human therapy of such ribozymes whether produced synthetically or as expressed by such transformed cells. See Chang, et al. Clinical Biotechnology 2:23-31 (1990) which is incorporated herein by reference.

SUMMARY OF THE INVENTION

The production of large amounts of specifically targeted ribozymes for therapeutic use is problematical. This invention provides a novel method for the large scale production of any ribozyme by expression of a stable RNA molecule into which catalytic RNA has been inserted. Any stable RNA can be utilized. In the preferred practice of the invention, E. Coli is transformed by Tac promoter

-3-

driven 4.5 S RNA having a catalytic RNA sequence inserted therein. High levels of expression of such fusion RNAs can be achieved by varying the temperature of growth, using mutants of E. Coli or other bacteria which are defective in certain enzymatic activities such as RNase III, inducing expression with isopropyl thio β galactopyranoside (IPTG) in an appropriate host, preparing a set of nested deletions within the 4.5 S or similar gene to eliminate undesirable folding and by other standard molecular biological techniques.

The invention also includes synthetic genes which encode catalytic RNA, microorganisms transformed with such genes, the catalytic RNA containing expression products of such genes and the in vitro and therapeutic use of such expression products.

DESCRIPTION OF THE FIGURES

Figure 1 illustrates a vector containing a Tac promoter and a ribozyme fused to an E. Coli 4.5 S RNA gene.

Figure 2 illustrates the vector shown by Figure 1 with the transcriptional unit containing the ribozyme depicted by Figure 1.

Figure 3 is a Northern analysis showing the expression of an anti-HIV-1 gag ribozyme fused with 4.5 S RNA (lanes a and b) and 4.5 S RNA alone (lane c).

Figure 4 depicts an ethidium bromide stained gel of an acid-phenol extract of small RNAs overproduced by E. Coli transformants in accordance with the invention.

Figure 5 depicts a preparative gel including ethidium stained RNAs as shown by Figure 4. The arrowhead points to the fusion ribozymes.

-4-

Figure 6 is a composite depiction of the RNA folding of the ribozyme embedded in the 4.5 S transcript.

Figure 7 depicts a gel electrophoresis analysis demonstrating in vitro cleavage reactions using the E. Coli transformants produced by 4.5 S fusion ribozymes.

DETAILED DESCRIPTION OF THE INVENTION

Heretofore, the preferred way to make ribozymes for in vitro studies or delivery to cells in culture was to scale up an in vitro transcription reaction. This invention eliminates the need for in vitro transcription thus greatly reducing the cost of producing large quantities of ribozyme.

In its more generic embodiments, the invention provides a novel method for the large scale production of any ribozyme by the expression of a stable bacterial RNA molecule into which a ribozyme sequence has been inserted.

The stable RNA can be any RNA and the insert can be any ribozyme. More specifically, any small stable bacterial RNA for which a gene is available may be utilized. The RNA molecule is preferably small, i.e., contains from about 50 to 200 nucleotides and is preferably an E. Coli RNA. E. Coli 4.5 S RNA is used in the method presently deemed to be the best mode for the practice of the invention.

Among others, any of the ribozymes described in application Serial No. 369,489 or Serial No. 401,613 may be used. A strong prokaryotic promoter system is chosen to facilitate overproduction of the RNA-ribozyme fusion product. Vectors including the stable RNA, ribozyme and promoter are transformed into bacteria selected to abundantly produce such fusion products.

-5-

In general, consensus sequence promoters are useful in the practice of this invention. The Tac promoter system is preferred. The thermally inducible lambda pL promoter system may also be utilized.

Vectors comprising, in combination, a stable RNA, an inserted ribozyme, and an appropriate promoter and a transcriptional terminator are an important component of the invention. Such vectors are constructed and transformed into bacteria in known manner.

EXAMPLE I

The E. Coli 4.5 S RNA gene is described in the prior art. See Brosius, J., et al., Biological Chemistry 260:3539-3541 (1985). This gene includes an MluI restriction site. The anti-HIV-1 gag ribozyme gene having the sequence 5' GGATCCGCTTAATACTCTGATGAGTCCGTGAGGACGAAACGCT CTCGCACCGGATCC 3' is inserted into the MluI site of the 4.5 S RNA molecule to produce the Figure 1 construct in the following manner:

The plasmid pKK243-1 was cut at the unique MluI restriction sight in the 4.5 S gene sequence. The staggered ends created by this digestion were filled in by Klenow DNA polymerase and dephosphorylated with calf intestine phosphatase. Two synthetic DNA oligomers with 10 bases of complementary sequences at their 3' termini, GAGHAM 1(GGCCGGATCCGCTTAATACTCTGATGAGTCCGTGAGGAC) and 2(CCGGATCCGCGAGAGCGTTTCGTCTCACGG), were polymerized

-6-

into a double stranded fragment with Taq polymerase by mixing equimolar amounts together in a 50 μ liter reaction containing ca. 0.5 μ gms of each oligo, 125 μ moles each dNTP, Taq polymerase buffer (Cetus-Perkin Elmer) and 2.5 units of Taq polymerase.

A series of 10 amplification cycles was carried out using the following: 94C-1 min., 37C-1 min. and 72C-2 min. The polymerized fragment was phosphorylated, ligated with pKK243-1 vector and transformed into E. Coli strain MC 1061 by standard calcium chloride techniques. Transformants were screened for the ribozyme insert and clones harboring the insert were examined for expression using a Northern gel analysis (see Figure 3). Low molecular weight RNA was harvested from positive clones by the following method. Cells were suspended in cold 50 mM Tris pH 7.5, 5 mM MgCl₂ and acid phenol extracted twice, made to 0.3 M NaOAc pH 5.5 and precipitated with 3.5 volumes of ethanol. The pellet was washed with 70% ethanol and resuspended in sterile water. Aliquots of this RNA were run on a 6% acrylamide (20:1 acrylamide:bis) gel at 40 millamps in TBE buffer, transferred to Zeta Probe nylon filter by electroblotting at 90mamps overnight, and hybridized with radioactively labeled gag ribozymes specific probe GAGHAM 2. Such Northern analysis showed both precursor (lane c) and final products (lanes a and b, Figure 3). The clone with the highest expression was chosen for further study. Both the precursor and processed product were cultured in "L" broth at 37°C and monitored for ribozyme activity after they were purified by overnight diffusion elution from the acrylamide gel in 0.3 M NaOAc pH 5.5, 0.1% SDS. Following elution, the RNA was extracted 2 times with phenol, once with dichloromethane, ethanol precipitated, pelleted by centrifugation and washed

-7-

with 70% ethanol. The RNA was dissolved in water before use. RNA fragments were tested for in vitro cleavage activity by the methods described by Chang, et al., supra.

Figure 4 depicts an ethidium bromide stained gel of an acid-phenol extract of small RNAs by which 4.5 S (left lane) and 4.5 S with an 8bp insertion on the MluI site (right lane) which were overproduced by such transformants. The arrows indicate the ethidium bromide stained products (4.5 S) (4.5 S and 8pb insertion) derived from less than 1 ml of cell culture.

Figure 5 depicts a preparative acid-phenol extract of RNA from approximately 250 mls of cell. The arrow points to the 4.5 S gag ribozyme fusion transcript. This transcript was excised from the gel and shown to be catalytically functional (see Figure 7).

-8-

WHAT IS CLAIMED IS:

1. A synthetic gene which encodes catalytic RNA.
2. The vector depicted by Figure 1.
3. A mammalian or a bacterial cell transformed with a synthetic gene as defined by claim 1 or the vector defined by claim 2.
4. A bacterial cell transformed with a synthetic gene as defined by claim 1 or the vector defined by claim 2.
5. A vector comprising a Tac promoter and a ribozyme fused to an E. Coli 4.5 S RNA gene.
6. A vector as defined by claim 5 in which said ribozyme is an anti-HIV-1 gag ribozyme.
7. A vector as defined by claim 6 in which the anti-HIV-1 gag ribozyme gene has the sequence
5' GGATCCGCTTAATACTCTGATGAGTCCGTGAGGACGAAACGCT
CTCGCACCGGATCC 3'.
8. The expression product of a cell transformed with a vector comprising a Tac promoter and a ribozyme fused to an E. Coli 4.5 S RNA gene.
9. The expression product of claim 8 in which said cell is a bacterial cell.
10. The expression product as defined by claim 9 in which said bacterial cell is an E. Coli cell.
11. The expression product as defined by claim 9 or claim 10 in which said ribozyme is an anti-HIV-1 gag ribozyme.
12. The expression product as defined by claim 9 or claim 10 in which said ribozyme comprises the sequence
5' GGAUCCGCUUAAUACUCUGAUGAGUCCGUGAGGACGAAACGCU
CUCGCACCGGAUCCGCGUGUGCC 3'.
13. The method which comprises cleaving HIV-1 RNA by reaction with an expression product as defined by claim 9 or claim 10.

1/5

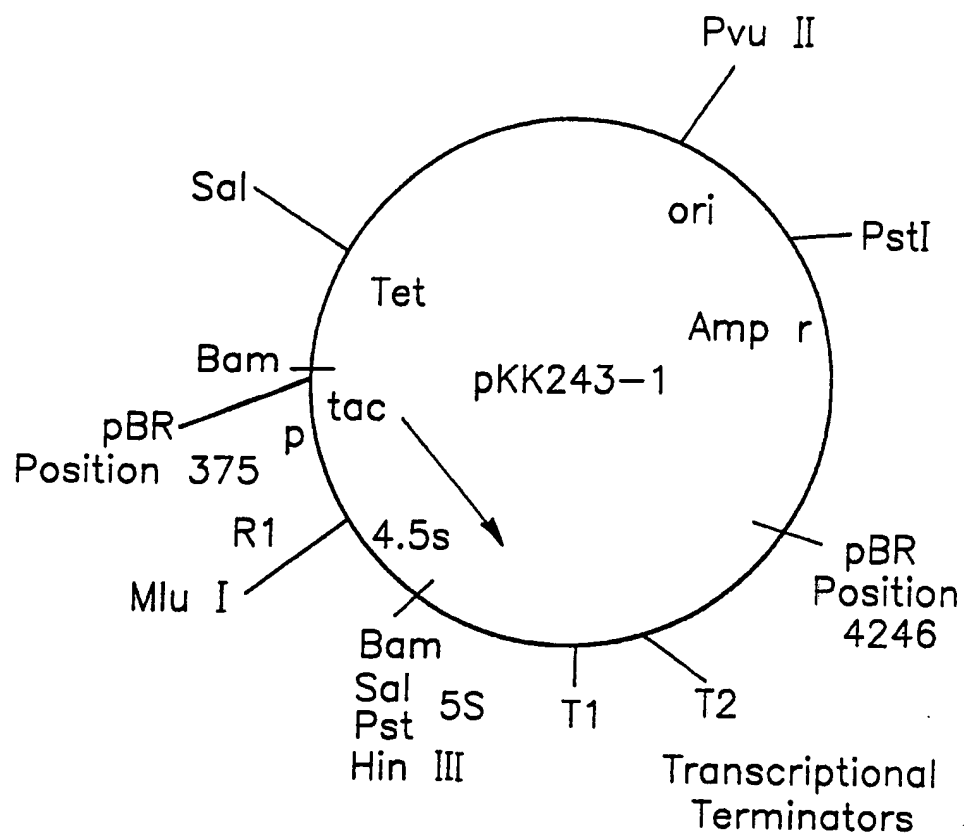


FIG. 1

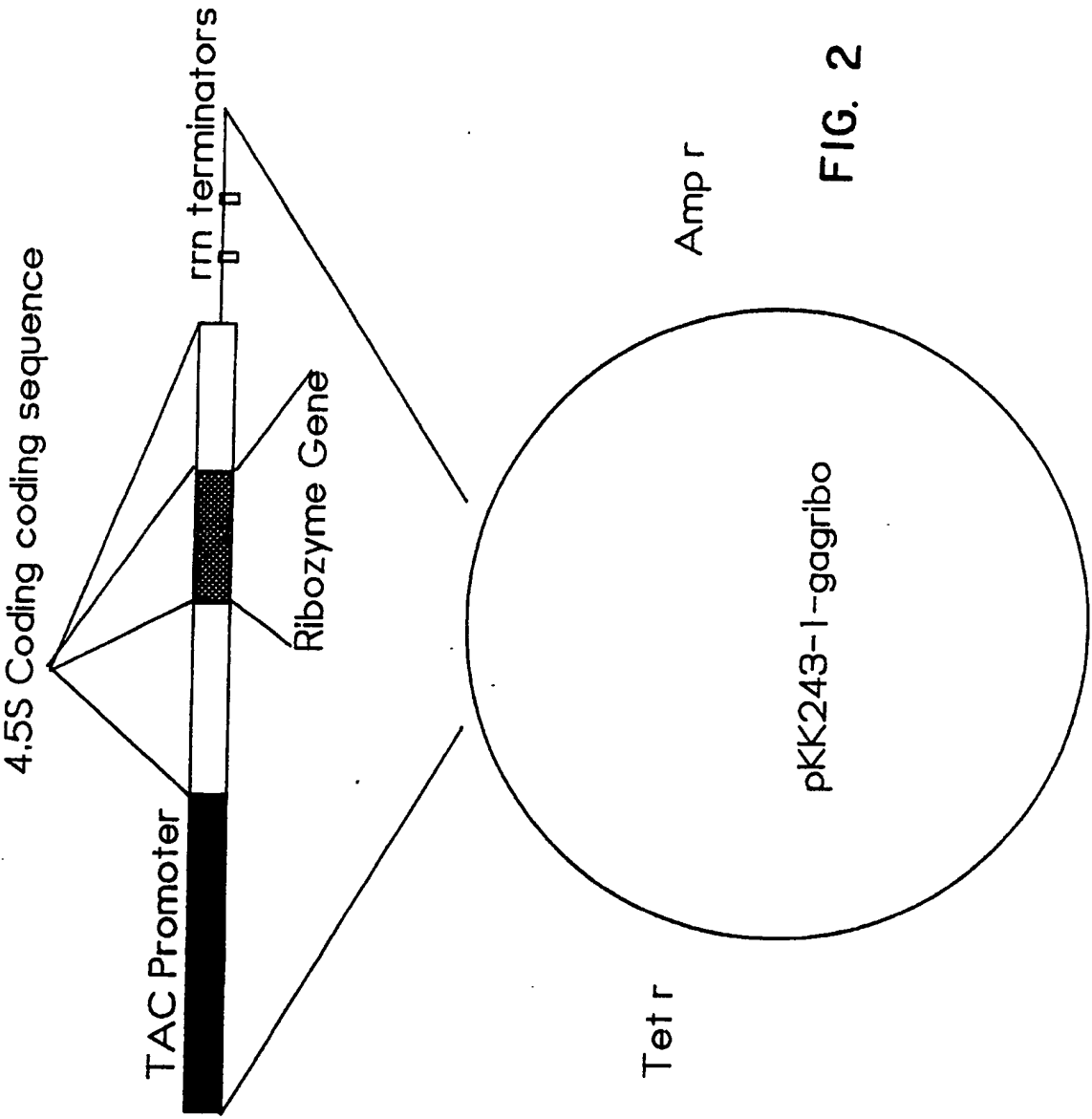


FIG. 2

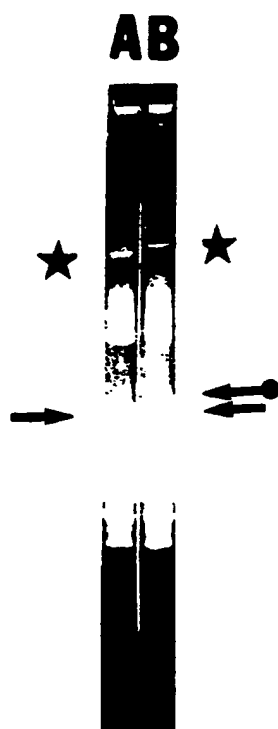


FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06032

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): C07H 15/12; C12Q 1/68; C12P 21/06, 21/02, 21/04, 19/34; C12N 15/00, 1/20		
U.S.C1.: 536/27; 435/6, 69.1, 70.3, 71.2, 91, 172.1, 172.3, 252.3, 252.33		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.C1	536/27; 435/6, 69.1, 70.3, 71.2, 91, 172.1, 172.3, 252.3, 252.33	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 14		
Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X	Science, volume 228, No. 4202, issued 24 May 1985 (Washington, DC) M. Baer, et al., "A. Catalytic RNA and Its Gene from <u>Salmonella Typhimurium</u> ", see pages 999-1002.	1
Y	Proc. Natl. Acad. Sci, volume 86, issued December 1989 (USA), Cameron, et al., "Specific Gene Suppression by Engineered Ribozymes In Monkey Cells", see pages 9139-9143.	2,3,5,8
A	Chemical Abstracts, volume 112, no. 7, issued 12 February, 1990, (Columbus, Ohio, U.S.A.) Gerlach, et al., "Synthetic Ribozymes for <u>in vivo</u> Inactivation of Prokaryotic or Eukaryotic RNA Transcripts" see page 337, column I, See abstract. no. 51284; , Eur. Pat Appl. EP 321,201,21 June 1989.	1-13
<p>* Special categories of cited documents: 15</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search *		Date of Mailing of this International Search Report *
04 January 1991		06 FEB 1991
International Searching Authority *		Signature of Authorized Officer 19
ISA/US		John W Rollins

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁴ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
A	Chemical Abstracts, Volume 110, no. 21, issued 22 May 1989 (Columbus, Ohio, U.S.A.) Cech, et al., "RNA Ribozyme Polymerase, Dephorylase Restriction Endoribonuclease and methods for their manufactors". See page 226, column 2, the abstract no. 187321K, PCT Int. Appl. W088 04,300, June 1988.	1-13
A	Proc. Natl. Acad. Sci., Volume 84 issued December 1987 (U.S.A.) Kim, et al., "Three-dimensional Model of the Active Site of the Self-splicing rRNA Precursor of Tetrahymena", see pages 8788-8792.	1-13
A	EMBO Journal. volume 8, no. 12 issued 1989, Cotten, et al., "Ribozyme Mediated Destruction of RNA <u>in vivo</u> ", see pages 3861-3866.	1-13